

# High postnatal lethality and testis degeneration in retinoic acid receptor $\alpha$ mutant mice

(vitamin A/gene targeting/spermatogenesis)

THOMAS LUFKIN\*, DAVID LOHNES, MANUEL MARK, ANDRÉE DIERICH, PHILIPPE GORRY,  
MARIE-PIERRE GAUB, MARIANNE LEMEUR, AND PIERRE CHAMBON†

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Médecine, 11, rue Humann 67085 Strasbourg Cedex, France

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**ABSTRACT** Retinoic acid (RA) plays a critical role in normal development, growth, and maintenance of certain tissues. The action of RA is thought to be mediated in part by the three nuclear receptors (RAR $\alpha$ , - $\beta$ , and - $\gamma$ ), each of which is expressed as multiple isoforms. To investigate the function of the RAR $\alpha$  gene, we have disrupted, in the mouse, the whole gene or the isoform RAR $\alpha$ 1. Although RAR $\alpha$ 1 is the predominant isoform and is highly conserved among vertebrates, RAR $\alpha$ 1-null mice appeared normal. However, targeted disruption of the whole RAR $\alpha$  gene resulted in early postnatal lethality and testis degeneration. These results, showing that RAR $\alpha$  is indeed involved in the transduction of the RA signal, also suggest an unexpected genetic redundancy.

Feeding animals a vitamin A (retinol)-deficient diet has shown that this vitamin plays a critical role in growth, maintenance of numerous tissues, and overall survival (1, 2). In addition, offspring of vitamin A-deficient dams exhibit a number of developmental defects (3). Most effects of vitamin A deficiency can be prevented or reversed by retinoic acid (RA) (4, 5). The teratogenicity of maternal RA administration and the effects of topical application of RA have further supported the idea that RA may play an important role in morphogenesis (6–8). It is thought that the effects of the RA signal are mediated through two families of receptors which act as inducible transcriptional regulatory proteins and belong to the superfamily of nuclear receptors. The three retinoic acid receptors (RAR $\alpha$ , - $\beta$ , and - $\gamma$ ) and their isoforms bind all-*trans*- and 9-*cis*-RA, while the three retinoid X receptors (RXR $\alpha$ , - $\beta$ , and - $\gamma$ ) bind only 9-*cis*-RA (refs. 9 and 10 and references therein). The high degree of conservation of a given receptor (or isoform) across vertebrates, and their specific patterns of expression during embryogenesis and in adult tissues, has suggested that each of the receptors performs a specific function (9). In this respect the RAR $\alpha$  gene is unique among the RARs in being almost ubiquitously expressed in embryonic and adult tissues (11–13). The transcripts of the major RAR $\alpha$  isoform, RAR $\alpha$ 1, whose promoter region resembles that of a housekeeping gene, are widely distributed, whereas the transcript distribution of the less abundant, RA-inducible isoform RAR $\alpha$ 2 appears to be more restricted (11, 14). To investigate the function of RAR $\alpha$ , we have deleted, in the mouse, either the whole gene or specifically the RAR $\alpha$ 1 isoform.

## MATERIALS AND METHODS

**Gene Targeting of RAR $\alpha$  and RAR $\alpha$ 1.** For full RAR $\alpha$  disruption, an 11-kb *EcoRI*–*Spe* I fragment (containing the

A2 and B regions) from  $\lambda$ G2mRAR $\alpha$  (11) was subcloned into the *EcoRI*/*Xba* I sites of pTZ18R to create p807. TGAGCGG was inserted after the CCA encoding the proline at aa 19 of the B region (11), creating an in-frame stop and a unique *Not* I restriction site to generate p819, into which the 1.7-kb *Not* I fragment containing the GTI-II enhancer-driven neomycin gene [purified from p581 (15)] was cloned to generate p826B1, which was linearized by cleavage at its single *Sal* I site and used for electroporation. p826B1 has 8 kb and 3 kb of genomic DNA 5' and 3', respectively, to the neomycin insertion. For RAR $\alpha$ 1 isoform disruption, a 9-kb *EcoRV*–*Sal* I fragment from  $\lambda$ G1mRAR $\alpha$  was subcloned into pBluescript KS(+) (Stratagene) to generate pD182, which was partially digested at the *Kpn* I site (at codon 19 of the A1 region), into which site was subcloned the 1.4-kb *Kpn* I enhancerless Rous sarcoma virus TATA box-driven neomycin gene fragment [derived from p581 (15)] to generate pD183. pD183 was digested with *EcoRV* and ligated with the 2.3-kb GTI-II enhancer-driven herpes simplex virus thymidine kinase gene fragment [purified from p565 (15)] to generate pD209, which was linearized at its single *Spe* I site and used for electroporation. pD209 contains 8 kb and 1 kb of DNA 5' and 3', respectively, to the site of neomycin insertion. Genomic DNA extraction, Southern blotting, embryonic stem (ES) cell culture, generation of chimeras, and probe preparation were as described (15). Probes 1, 2, and 3 correspond to 1.4-kb *Xba* I–*Spe* I (from  $\lambda$ G2mRAR $\alpha$ ) and 0.8-kb *Bam*HI and 1.4-kb *Not* I–*Sma* I (from  $\lambda$ G1mRAR $\alpha$ ) fragments, respectively; both 2 and 3 were used in RAR $\alpha$ 1 disruption.

**Western Blot Analysis.** Embryos from RAR $\alpha$  heterozygote matings were removed at 13.5 days postcoitum (dpc), and the yolk sac was taken for DNA genotyping. Whole cell extracts from transfected COS-1 cells and nuclear extracts from embryos were prepared as described (16, 17). Sample denaturation, electrophoresis, transfer to nitrocellulose, blocking, and antibody probing were as described (16). Primary antibody was detected with protein A-coupled horseradish peroxidase followed by chemiluminescence reagents (Amersham). Rabbit polyclonal antibodies specific to RAR $\alpha$  [RP $\alpha$ (F)] and RAR $\beta$  [RP $\beta$ (F)2] were generated as described (17, 18).

## RESULTS

Two types of mutant alleles of the RAR $\alpha$  gene were created by homologous recombination using the “replacement” strategy (19) (Fig. 1a). The first mutant allele (termed RAR $\alpha$ )

Abbreviations: CRABP, cellular retinoic acid-binding protein; dpc, day(s) postcoitum; ES, embryonic stem; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

\*Present address: Brookdale Center for Molecular Biology, Mount Sinai Medical Center, 1 Gustave L. Levy Place, New York, NY 10029.

†To whom reprint requests should be addressed.

ES clones (KA3, KA5, and KA26) gave germ-line transmission. Mice heterozygous for either mutation were healthy and fertile. Intercrossing of heterozygous mice for either mutation produced homozygous offspring (Fig. 1b and below).

To verify that the *RAR $\alpha$*  and *RAR $\alpha$*  RNAs were functionally disrupted, we performed RNase protection assays using RNA from embryos at 13.5 dpc (a time at which *RAR $\alpha$*  RNA is abundantly expressed; see ref. 13). Wild-type embryos and embryos heterozygous for the *RAR $\alpha$*  mutation expressed the two major *RAR $\alpha$*  isoforms (*RAR $\alpha$* 1 and *RAR $\alpha$* 2; Fig. 1c, lanes 2 and 3). However, in the *RAR $\alpha$*  homozygotes, only the mutant form of *RAR $\alpha$*  RNA was present (*RAR $\alpha$* mut; Fig. 1c, compare lane 4 with lanes 2 and 3). Similarly, for the *RAR $\alpha$* 1 mutation, wild-type and hetero-

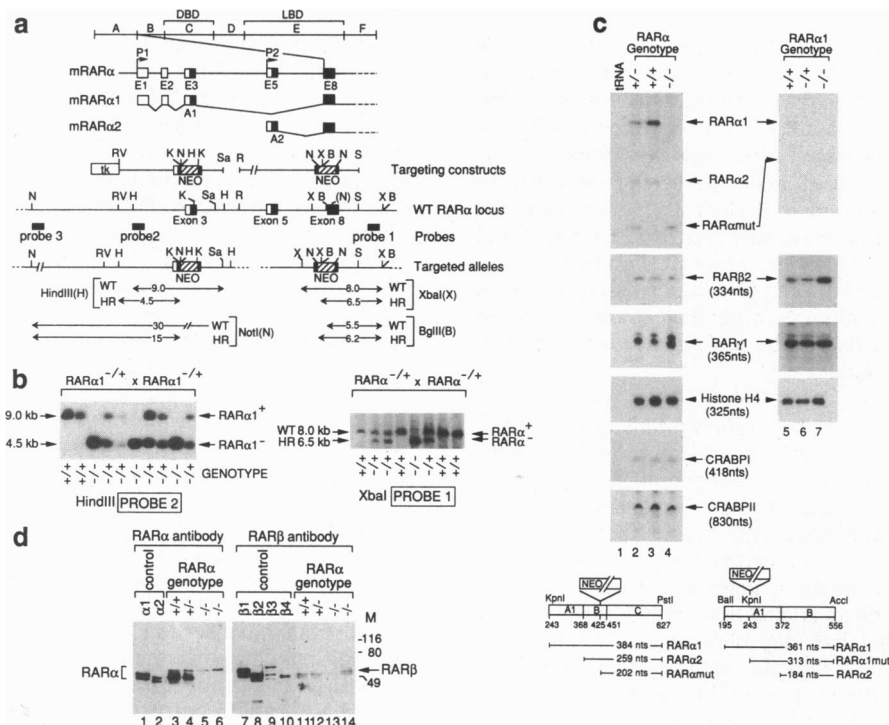


FIG. 1. (a) RAR $\alpha$  targeting constructs, wild-type RAR $\alpha$  locus, and disrupted alleles. The various regions of the RAR $\alpha$  protein (A-F), the DNA-binding domain (DBD), and the ligand-binding domain (LBD) are indicated at the top (9). The alternative promoter (P1 or P2) usage and alternative splicing of exons (E1-E8), which generate the  $\alpha 1$  and  $\alpha 2$  isoforms, are also shown. mRAR, mouse RAR. The two targeting constructs are drawn above the wild-type (WT) RAR $\alpha$  locus. The RAR $\alpha 1$  targeting construct (left) has the neomycin-resistance gene (NEO) inserted into the A1 region encoded by exon 3 (E3) and has a herpes simplex virus thymidine kinase gene (tk) at its 5' end. The RAR $\alpha$  targeting construct (right; note that it does not include tk) has NEO inserted into the B region, which is encoded by exon 8 (E8) (11). Plasmid vector sequences are not shown. The structure of the targeted alleles and the restriction enzyme digests and DNA probes used for Southern blotting are indicated. B, *Bgl* II; H, *Hind* III; K, *Kpn* I; N, *Not* I; R, *Eco* RI; RV, *Eco* RV; S, *Spe* I; Sa, *Sal* I; X, *Xba* I. (b) Southern blots of offspring from intermatings of mice heterozygous for either RAR $\alpha$  or RAR $\alpha 1$  disruptions. The positions of the wild-type (+) and mutant (-) alleles are indicated, as well as their size. Offspring genotypes are indicated below the lanes: +/+, wild type; +/-, heterozygote; -/-, homozygote. Probes 1 and 2 correspond to the probes shown in a. (c) RNase protection analysis of RNA from 13.5-day embryos that were +/+, +/-, or -/- for either the RAR $\alpha$  (lanes 1-4) or the RAR $\alpha 1$  (lanes 5-7) disruption. Fifty micrograms of RNA purified as described (20) was used per hybridization at 55°C for 8-12 hr. Probe preparation and hybridization reactions were as described (22). Templates for synthesis of labeled RNA probes were prepared by subcloning the following cDNA fragments: for RAR $\alpha$  in the RAR $\alpha$  and RAR $\alpha 1$  disruptions, the 384-bp *Kpn* I-*Pst* I RAR $\alpha 1$  and the 361-bp *Bal* I-*Acc* I RAR $\alpha 1$  cDNA fragments, respectively (11); for RAR $\beta$ , the 334-bp *Pst* I RAR $\beta 2$  cDNA fragment (23); for RAR $\gamma$ , nt 235-600 of RAR $\gamma 2$  (21) were obtained by PCR amplification; for cellular retinoic acid binding protein I (CRABPI), the 418-bp *Eco* RI-Ava I cDNA fragment (24); for CRABPII, the 839-bp *Eco* RI-*Hind* III cDNA fragment (25); for histone H4, the 630-bp *Eco* RI-*Hind* III genomic fragment (a gift of R. Grosschedl, Howard Hughes Medical Institute, University of California, San Francisco). All RAR cDNA subclones contained an isoform-specific A region and common B and C regions. The identities of the protected fragments (RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ , CRABPI, CRABPII, and histone H4) are indicated by the arrows. In the case of RAR $\beta$  and RAR $\gamma$ , only the protected fragments corresponding to the major isoforms RAR $\beta 2$  and RAR $\gamma 1$  are shown; similar results were obtained for the other isoforms (RAR $\beta 1$ , - $\beta 3$ , and - $\beta 4$  and RAR $\gamma 2$ ; data not shown). The source of RNAs used in the protection assays was as follows: lane 1, tRNA (negative control); lane 2, RAR $\alpha$  +/-; lane 3, +/+; lane 4, RAR $\alpha$  -/-; lane 5, +/-; lane 6, RAR $\alpha 1$  +/-; lane 7, RAR $\alpha 1$  -/-. (d) Western blot analysis of nuclear proteins isolated from RAR $\alpha$  +/+, +/-, and -/- embryos at 13.5 dpc. Lanes 1 and 2, transfected COS-1 cells expressing RAR $\alpha 1$  and RAR $\alpha 2$ , respectively; lane 3, RAR $\alpha$  +/- embryos; lane 4, RAR $\alpha$  -/- embryos; lanes 5 and 6, RAR $\alpha$  -/- embryos; lanes 7-10, transfected COS-1 cells expressing RAR $\beta 1$ , - $\beta 2$ , - $\beta 3$ , or - $\beta 4$ , respectively; lane 11, RAR $\alpha$  +/+ embryos; lane 12, RAR $\alpha$  +/- embryos; lanes 13 and 14, RAR $\alpha$  -/- embryos. RAR $\alpha$ -specific and RAR $\beta$ -specific antisera were used in lanes 1-6 and 7-14, respectively. One to 5  $\mu$ g of COS-1-transfectant protein extract and 70  $\mu$ g of embryo nuclear protein extract were loaded per lane (except in lanes 5 and 13 where  $\approx 35$   $\mu$ g of protein was loaded). Upper band in lanes 3-6 corresponds to a nonspecific immunoreaction.

zygous embryos expressed wild-type *RARα1* and *-α2* RNAs (Fig. 1c, lanes 5 and 6, and data not shown), but only the mutant form of the *RARα1* RNA (*RARα1mut*) and *RARα2* RNA were detectable in the homozygotes (Fig. 1c, compare lane 7 with lanes 5 and 6, and data not shown). RNA levels of the two other RARs (*RARβ* and *RARγ*) did not vary among wild-type, heterozygous, and homozygous embryos for either mutation (Fig. 1c), indicating that *RARα* does not play a unique role in controlling *RARβ* and *RARγ*, whose expression is enhanced by RA (refs. 27 and 28 and references therein). Western blot analysis was also used to verify the *RARα* disruption. Antibodies directed against the F region of *RARα* readily detected *RARα* in extracts from wild-type and heterozygous 13.5-dpc embryos (Fig. 1d, lanes 3 and 4), whereas it could not be detected in *RARα* homozygotes (lanes 5 and 6). Immunoblotting with antibodies directed against the F region common to all *RARβ* isoforms did not reveal any significant variation (within the sensitivity of the assay) among the same protein extracts (Fig. 1d, lanes 11–14).

The viability of *RARα*- and *RARα1*-null homozygotes was determined by intercrossing heterozygous animals. *RARα1*-null homozygotes represented ≈25% of the offspring at all gestational and postnatal stages (Table 1 and data not shown). Further, *RARα1* homozygotes were fertile and intercrossing of homozygotes generated litters of *RARα1*-null animals which appeared healthy, fertile, and phenotypically normal. Histological analysis and whole-mount skeletal staining did not reveal any detectable malformations in the *RARα1*-null homozygotes. When analyzed during gestation or after cesarean delivery at 18.5 dpc, *RARα*-null homozygotes also represented 25% of all embryos and fetuses, demonstrating that full disruption of the *RARα* gene is not embryonic lethal. No obvious malformations or lesions were macroscopically or histologically detected. However, genotyping of animals as soon as 12–24 hr postpartum showed a 60% deficiency of *RARα*-null homozygotes. Yet all cesarean-delivered pups survived up to 24 hr when isolated from their dams, indicating that up to 60% of the homozygotes had been preferentially cannibalized by their mother during this brief period (see Table 1, 1 day postpartum). Analysis at later times showed a continuing decrease in *RARα*-null homozygotes relative to wild-type and heterozygous littermates, with homozygotes representing only 3% of the total population at 1–2 months (Table 1). In fact, 75% of the *RARα*-null homozygotes which remained after 1 day disappeared during the next 1–2 months. Some of these mice showed a slower growth rate after 1–2 weeks and before death became emaciated and lethargic. No obvious malformations could be detected, with the exception that 60% of these homozygotes displayed webbed digits on both forelimbs and hindlimbs; however, the precise digits fused varied between individuals and between limbs within

the same animal. This interdigital webbing never regressed, and staining of bone and cartilage showed that it was restricted to soft tissues (data not shown). This phenotype was not seen in wild-type or *RARα*-heterozygous mice, whose digits become fully separated by 2 weeks of age.

The *RARα* homozygotes that survived for >2 months surprisingly appeared superficially normal, being similar in size to their wild-type or heterozygous littermates, but none of the five males tested (up to the age of 5 months) sired any offspring, even though caged with fertile wild-type females. The testes of four of these males at 4–5 months showed severe degeneration of the germinal epithelium (Fig. 2). The parenchyma of the testes of *RARα*-null homozygotes displayed patchy lesions of the seminiferous tubules, with rare tubules which appeared histologically normal (T1, Fig. 2 *b* and *f*; compare with *a* and *d*), while adjacent tubules were markedly atrophic (T3, Fig. 2c) and/or mostly devoid of spermatogenic cells (e.g., spermatogonia, spermatocytes, spermatids and spermatozoa; T2 in Fig. 2 *b* and *c*; Fig. 2e). In addition, vacuolation was frequently seen within the cytoplasm of Sertoli cells (v, Fig. 2 compare *b* and *c* with *a*, and *e* with *d*), and cytoplasmic expansions of these cells often partially filled the lumen of the seminiferous tubules (T2, Fig. 2 *b*, *c*, and *e*). The lumen of the epididymal duct contained very few spermatozoa (Z, Fig. 2 *g* and *h*). Thus, spermatogenesis appeared to be drastically reduced in *RARα*-null homozygotes, although it was not totally abolished as indicated by the presence of a few spermatozoa in the seminiferous epithelium of rare tubules and in the lumen of the epididymal duct. In contrast no lesions were observed in the seminal vesicles and prostate (data not shown).

## DISCUSSION

Animals fed a vitamin A-deficient diet develop a syndrome which includes widespread substitution of keratinizing squamous epithelium for normal epithelium, atrophy of several glandular organs, eye lesions, testis degeneration, and emaciation (1, 2, 5). These animals eventually die. In addition, offspring of vitamin A-deficient females exhibit a broad array of abnormalities which mainly involve the eye, genitourinary tract, kidney, heart, and lung (3). Our results show that *RARα* plays a crucial role in transducing the RA signal in mice, since >90% of the *RARα*-null homozygotes died before the age of 2 months. As is the case for the vitamin A deficiency syndrome, some of these mice have a slower rate of growth and become emaciated, even though no specific lethal lesions can be identified macroscopically or histologically. Thus, *RARα* appears to be involved in the maintenance of some homeostatic processes, as has been inferred from its apparently ubiquitous expression in the adult animal (9, 11–13). Surprisingly, with the exception of testis degeneration, *RARα*-null homozygotes do not display any of the lesions associated with vitamin A deficiency. These observations suggest that the other RARs and/or RXRs (9) may mediate the retinoid signal in the events which are reflected by the occurrence of specific developmental abnormalities and postnatal lesions associated with vitamin A deficiency; alternatively, the other RARs and RXRs can substitute for *RARα* in the retinoid control of these events. Since the domains of expression of the other RAR and RXR transcripts are generally more restricted than that of *RARα* (refs. 9, 11–13, 30, and 31; P. Dollé, D. Décimo, and P.C., unpublished results), it appears either that their expression domains are wider than revealed by *in situ* hybridization or that in many locations, transcription of the *RARα* gene does not reflect an actual function of the receptor.

It has been claimed that retinoid deficiency leads to testis degeneration that cannot be reversed by RA administration, implying that retinol plays a unique role not only in vision but also in spermatogenesis (5, 32). The degeneration of the

Table 1. Viability of *RARα*- and *RARα1*-null offspring

Age	No. alive (ratio to wild type)		
	-/-	+/-	+/+
<i>RARα</i> <sup>+/+</sup> × <i>RARα</i> <sup>+/+</sup> offspring			
8.5–18.5 dpc	34 (0.9)	64 (1.6)	39 (1.0)
1 day postpartum	13 (0.4)	56 (1.6)	36 (1.0)
2 weeks	15 (0.2)	123 (1.9)	64 (1.0)
1–2 months	4 (0.1)	90 (2.0)	45 (1.0)
<i>RARα1</i> <sup>+/+</sup> × <i>RARα1</i> <sup>+/+</sup> offspring			
1–2 months	52 (0.9)	108 (1.9)	58 (1.0)

Genotypes of offspring from intermatings of either *RARα* or *RARα1* heterozygotes are given. Different litters were genotyped at the times shown. Hence, one can compare only rows of numbers. For each time point, the distribution of offspring is shown in parentheses relative to wild type (+/+). The percentage of *RARα*-null (-/-) homozygotes decreases with time. This decrease plateaus at 1–2 months with only 10% of the *RARα* homozygotes still alive.

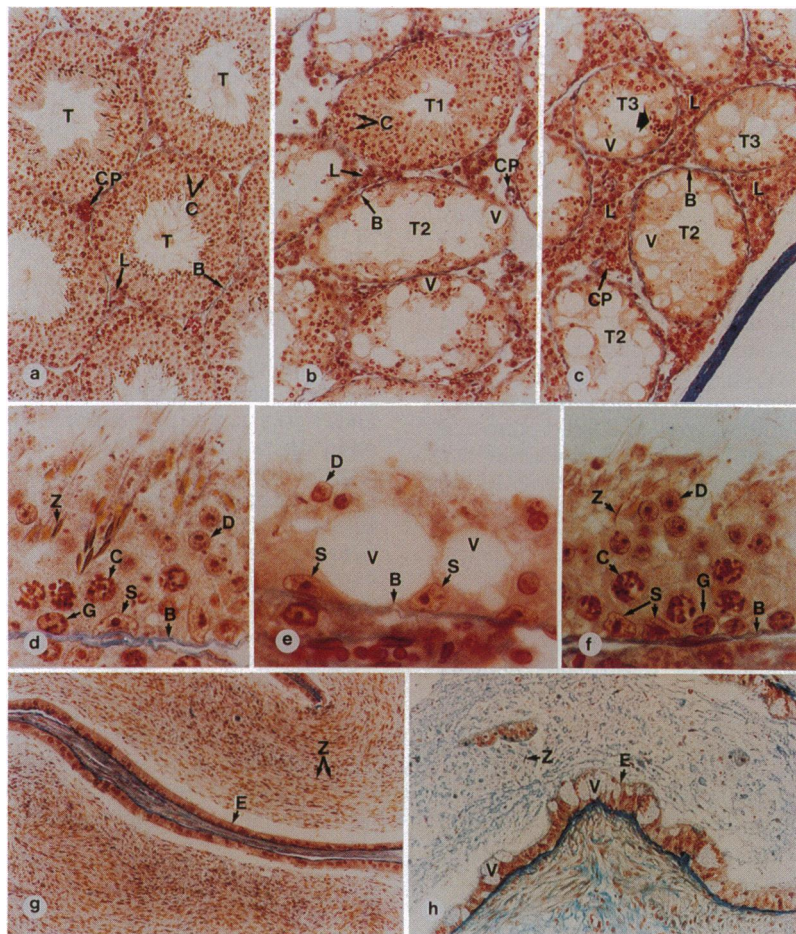


FIG. 2. Degenerative lesions in testes of 4- to 5-month-old  $RAR\alpha$ -null homozygotes. Comparison of histological sections through the testes (*a-f*) and epididymal ducts (*g-h*) of wild-type (*a, d, and g*) and  $RAR\alpha$ -null homozygotes (*b, c, e, f, and h*). (*a*) Parenchyma of wild-type testis is composed of seminiferous tubules (T) with active spermatogenesis and intertubular spaces containing capillaries (CP) and Leydig cells (L). The aspect of the seminiferous epithelium (or germinal epithelium) varies between tubules at different stages of the spermatogenic cycle; however, all tubules contain primary spermatocytes (C), each of which will eventually yield four spermatozooids. B, basement membrane. (*b* and *c*) Parenchyma of testis of  $RAR\alpha$  homozygote shows a patchy pattern of seminiferous tubule lesions. These cover a wide spectrum, ranging from rare tubules with complete spermatogenesis (e.g., T1) to tubules containing only Sertoli cells (e.g., T2) which may be enlarged, thus filling the tubules (e.g., T2 in *c*). A majority of tubules lack primary spermatocytes (C). In addition, the seminiferous epithelium shows numerous large, clear, rounded spaces (vacuole-like, V) and occasional clusters of degenerating spermatogenic cells (large arrow in *c*). In the intertubular spaces, focal hyperplasia of the Leydig cells (L) is observed between atrophic seminiferous tubules (*c*). This hyperplasia is likely to result from the decrease in tubular diameter (compare T3 in *c* with T in *a*; see ref. 29). (*d-f*) High-magnification micrographs of the walls of seminiferous tubules. (*d*) In wild-type testis, the seminiferous epithelium consists of supporting cells, the Sertoli cells (S), and spermatogenic cells. The spermatogenic cells proliferate from stem spermatogonia (G), located in contact with the basement membrane (B), and differentiate from the periphery toward the lumen of the seminiferous tubules. This process yields different ontogenetically related cell types arranged in concentric layers—i.e., spermatogonia (G), primary spermatocytes (C), round spermatids (D), and maturing spermatozooids (Z). (*e* and *f*) Two different aspects of the seminiferous epithelium in  $RAR\alpha$ -null homozygotes. Most frequently, the early stages of spermatogenic cell differentiation (e.g., spermatogonia and primary spermatocytes) are missing (*e*) (in such a degenerate epithelium, spermatogenesis no longer occurs). In rare cases, all stages of spermatogenic cell differentiation, including the round spermatids (D) and maturing spermatozooids (Z), are seen (*f*). (*g*) Section through the tail of a wild-type epididymal duct; spermatozooids (Z) fill the lumen. (*h*) Section through the tail of an epididymal duct of a  $RAR\alpha$  homozygote; the lumen of the duct contains acidophilic (blue) material which is also present within large vacuoles (V) in the epithelium lining the duct (E), possibly as a consequence of extensive cellular absorption; spermatozooids (Z) are occasionally identified in the lumen. Organs were immersed-fixed in Bouin's fluid. Paraffin sections (5  $\mu$ m thick) were stained with Groat's hematoxylin and Mallory's trichrome. [ $\times 217$  (*a-c, g, and h*);  $\times 1085$  (*d-f*).]

germinal epithelium in  $RAR\alpha$ -null homozygotes is similar, if not identical, to that observed in males kept on a vitamin A-deficient diet (32, 33). Thus, our results strongly suggest that RA, and not retinol, is required for the maintenance of spermatogenesis. This conclusion is supported by the observation that repeated administration of high doses of RA can restore spermatogenesis in males fed a vitamin A-deficient diet (34) and by the presence of CRABPI and  $RAR\alpha$  in germ cells (35–37). The retinol requirement may reflect a blood–testis barrier preventing RA delivery to the adluminal compartment of the seminiferous tubules (32, 38). Cellular retinol-binding protein I-containing Sertoli cells which form this blood–testis barrier may normally convert retinol to RA for

delivery to the germ cells (35, 38). It has been proposed (35) that the blood–testis barrier is less restrictive in birds, where the typical mammalian Sertoli–Sertoli cell junctions are absent and spermatogenesis can be restored by RA in retinol-deficient animals (39).

The selective cannibalism of  $RAR\alpha$ -null newborns indicates that they exhibit an abnormal phenotype which we have not yet recognized but is recognized by their mothers. Not all the null newborns are eaten, which suggests that the “cannibalizable” phenotype has a variable penetrance that may be related to the nonhomogeneous genetic background of the null homozygotes. Also, the variable penetrance of the webbed-digit phenotype, which appears to be associated with

early death (2–3 weeks), and the longer survival of a small fraction of RAR $\alpha$ -null homozygotes ( $\geq 2$  months) may have a similar origin. Moreover, the webbing is often different when pairs of limbs of a given animal are compared. This variability, which cannot be accounted for by variations in the genetic background, is most probably related to the stochastic nature of gene activity (40) in the cells which give rise to bilateral and symmetrical structures within an animal.

The transcripts of the major RAR $\alpha$  isoform, RAR $\alpha 1$ , are ubiquitously expressed, whereas those of the second most common isoform, RAR $\alpha 2$ , could not be detected by *in situ* hybridization (refs. 11 and 14; E. Ruberte, P. Dollé, and D. Décimo, personal communication). It is therefore surprising that, in agreement with a recent report (41), RAR $\alpha 1$ -null homozygotes did not exhibit any of the abnormalities seen in RAR $\alpha$ -null mice. This may mean that RAR $\alpha 1$  and RAR $\alpha 2$  are largely functionally redundant and that RAR $\alpha 2$  may have a wider domain of expression than suggested from the *in situ* hybridization data. (Note, however, that the global expression of RAR $\alpha 2$  was not altered in RAR $\alpha 1$ -null homozygotes.) Alternatively, in most places RAR $\alpha 1$  transcription may not reflect an actual function of this isoform, and RAR $\alpha 2$  may fulfill most of the function of the RAR $\alpha$  gene. In any event, the high degree of conservation of RAR $\alpha 1$  across vertebrates indicates that this isoform must perform some specific function conferring a selective advantage (see refs. 42 and 43).

The almost ubiquitous expression of RAR $\alpha$  (mainly RAR $\alpha 1$ ) has suggested that it may mediate RA induction of the RA-responsive RARs—i.e., RAR $\alpha 2$ , RAR $\beta 2$ , and RAR $\gamma 2$  (9, 14, 23, 28). No change in the level of their expression was seen in RAR $\alpha$ - or RAR $\alpha 1$ -null homozygotes, indicating that if RAR $\alpha$  is involved in these inductions, its function must be redundant. Note also that the level of expression of the RA-responsive CRABP II gene (25, 44) was unchanged in RAR $\alpha$ -null homozygotes (Fig. 1c). RAR $\alpha$  is also the only RAR whose expression could be detected in the precise rhombencephalic region (see ref. 9 for review) where the product of the RA-inducible homeogene *Hoxa-1* (*Hox-1.6*) (45) plays a critical role during morphogenesis (15, 46). No hindbrain or inner ear lesions resembling those resulting from *Hoxa-1* deletion (refs. 15 and 46; M.M., T.L. and P.C., unpublished data) were seen in RAR $\alpha$ -null homozygotes. Therefore, it appears either that *Hoxa-1* expression is not critically dependent on RA induction in the animal or that other RARs or RXRs whose expression has not been detected by *in situ* hybridization in this region of the hindbrain could control the RA responsiveness of *Hoxa-1*. Disruption of other RARs and RXRs must be performed to investigate these possibilities and the extent of redundancy between these receptors.

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